IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In re application of: A.P. Wolffe *et al.* Application No.: 09/844,501 Filed: April 27, 2001 For: DATABASES OF REGULATORY SEQUENCES; METHODS OF MAKING AND USING SAME	Examiner: Jeffrey N. Fredman Group Art Unit: 1637 Confirmation No.: 9055

REPLY TO EXAMINER'S ANSWER

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I hereby certify that this correspondence is being deposited with the United States Postal Service as first class mail in an envelope addressed to: Commissioner for Patents, Alexandria, VA 22313 on **January 27, 2005**.

1/27/05 Michelle Hobson

Date Signature

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Mail Stop Appeal Brief
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Alexandria, VA 22313

Sir:

INTRODUCTION

Appellants submit this reply brief on appeal in accordance with 37 C.F.R. § 1.193(b)(1). An Examiner's Answer was mailed on November 17, 2004. In light of the record in this case and following remarks, Appellants respectfully request that the decision of the Examiner be reversed.

REMARKS

The Examiner has maintained the rejections of all pending claims as allegedly obvious over U.S. Patent No. 5,635,355 ("Grosveld") alone or in view of the NEB catalog, U.S. Patent No. 5,500,356 ("Li") or U.S. Patent No. 6,444,421 ("Chung") under 35 U.S.C. § 103(a).

For the reasons of record and those previously set forth in the Appellants' Brief on Appeal, the claims on appeal are patentable over the combination of cited references because there is no teaching, suggestion or motivation within these references to support the assertions made by the Examiner. No reference alone, nor any combination of any of these references would result in the claimed methods.

Appellants address the issues raised in the Examiner's Answer in turn below.

1. Statement of the Issue

The Examiner's Answer stated that the issue is whether "intrinsic evidence provided by the express broad definition of the claim term 'library' in the specification supports the reasonable interpretation that the Grosveld references teaches a 'library' within the scope of the claimed invention." (Examiner's Answer, page 10)

The Examiner's statement of the issue is not accurate. The issue remains whether the claims, when properly construed, are obvious over Grosveld. For the reasons of record and reiterated herein, Appellants again submit that Grosveld fails to render the claims obvious.

The Examiner has constructed a rather tortuous obviousness argument that is based on a picking and choosing of various portions of Grosveld's disclosure, combined with a strained definition of the term "library" as encompassing a single sequence. However, regardless of the definition of the term "library," the record shows that no motivation exists for one of skill in the art to combine the teachings from Grosveld in the way that has been set forth by the Examiner; moreover, said combination fails to suggest the claimed methods. Thus the *prima facie* assertion of obviousness fails. *See* Section 2 below.

Furthermore, and again contrary to the Examiner's assertions, Appellants have provided evidence that their use of the term "library" is consistent with its ordinary meaning in the art, and that the specification shows no intent of the part of Appellants to impart a novel meaning, or one contrary to its normal meaning in the art, to the term. *See* Section 3. Appellants have also provided evidence establishing that the Examiner's attempted definition of the term is inconsistent with its ordinary meaning in the art. *See* Section 3.

2. *Prima Facie* Case

The Examiner maintained that Appellants did "not dispute that Grosveld teaches the steps as illustrated." (Examiner's Answer, page 10). In support of this assertion, the Examiner's Answer states, on page 10:

> Grosveld teaches each and every limitation of the invention of claim 123, with elements (a)-(d) of claim 123 found in column 8, lines 1-25 and elements (e)-(f) found in column 15, lines 43-47 and in claim 1 at column 21. ...
>
> While the Appellant disputes the prima facie case, the Appellant does not dispute that Grosveld teaches the steps as illustrated. What Appellant does dispute is whether Grosveld meets the preamble limitation that a "library" is prepared.

In fact, Appellant has consistently and strongly disputed the assertion that Grosveld teaches the steps of independent claim 123. Indeed, Appellants have previously pointed out that the fragments cloned by Grosveld are different from the fragments cloned in the claimed method. Whereas the fragments cloned by Grosveld are individual purified fragments containing a DNAse hypersensitive site within the fragment (*see* Grosveld at column 15, for example), an entire genome's worth of DNA is cleaved in the practice of the claimed method. *See, e.g.,* independent claim 123, which recites the provision of a cell nucleus [step (a)] and contact of the nucleus with a first enzyme; [step (b)]).[1] The resulting collection of genomic DNA fragments [step (d)] is then subjected to a cloning step [step (e)] such that fragments containing one end defined by cleavage with

[1] Note also that the claims do not require that the first enzyme be DNase I (see, *e.g.*, claim 127) and therefore are not restricted to cleavage at DNase hypersensitive sites.

the first enzyme are selectively cloned. *See* also Response filed December 17, 2003, resubmitted February 26, 2004.

Above and beyond the fact that the portions of Grosveld's disclosure chosen by the Examiner do not suggest the claimed methods, no plausible motivation for their combination has been advanced. The Examiner has alleged that Grosveld provides the motivation because the reference discloses ligation into a vector of fragments that had previously been characterized as containing DNase I hypersensitive sites, and because such vectors can be used to provide integration site-independent expression of a transgene that is present in the same vector. *See*, for example, Office Action of September 29, 2003, page 6. However, the combination of Grosveld's identification of DNase hypersensitive sites, and their use in a vector containing a transgene to obtain site-independent expression of the transgene after random integration of said vector into a genome, in no way suggests a method for obtaining a collection of regulatory sequences by selective cloning of enzymatically-cleaved genomic DNA, as claimed.

Faced with a similar issue (namely, an obviousness rejection based on a number of statements from a single reference), in *In re Kotzab* 217 F.3d 1365, 55 USPQ2d 1313 (Fed. Cir. 2000), the Federal Circuit reversed the finding of obviousness. In reversing, the Federal Circuit held that statements in the prior art must be considered in the context of the teaching of the entire reference, and that rejection of claims cannot be predicated on mere identification in a reference of individual components of claimed limitations. *Kotzab,* 55 USPQ2d at 1316-1317. Indeed, the Court specified that particular findings must be presented as to why a skilled artisan, with no knowledge of the claimed invention, would have selected those components for combination <u>in the manner claimed</u>. *Id.* The Federal Circuit also stated that mere identification of prior art statements that, in the abstract, appeared to suggest claimed limitations does not establish a *prima facie* case of obviousness without a finding as to a specific understanding or principle within the knowledge of the skilled artisan that would have motivated one with no knowledge of the invention to make the combination in the manner claimed (*In re Kotzab,* 55 USPQ2d at 1317):

> While the test for establishing an implicit teaching, motivation or suggestion is what the combination of these two

> statements [in the reference] would have suggested to those of ordinary skill in the art, the two statements cannot be viewed in the abstract. Rather, they must be considered in the context of the teaching of the entire reference. Further, a rejection cannot be predicated on the mere identification [in the reference] of individual components of claimed limitations. Rather, particular findings must be made as to the reason the skilled artisan, with no knowledge of the claimed invention, would have selected these components for combination in the manner claimed.

Applying the law as set forth by the Court in *Kotzab,* Appellants note that Grosveld's disclosure is directed to construction of vectors, comprising a gene sequence, to be used for transfection of mammalian cells and integration into the genome of said cells, so as to obtain expression of the gene. *See* Grosveld Abstract (cover page) and "Field of the Invention" (Column 1). The Examiner has not explained how, in this context, one of skill in the art would be led to pick and choose those portions of Grosveld's disclosure that the Examiner alleges would lead to the presently-claimed methods for obtaining collections of regulatory sequences. As such, the obviousness rejection is unsustainable.

Furthermore, the obviousness rejection cannot be maintained because the Examiner has failed to identify a specific understanding or principle within the knowledge of the skilled artisan that would have motivated him or her to combine the various teachings scattered throughout Grosveld's disclosure in a way that would arrive at the claimed subject matter. Although the teachings selected by the Examiner from Grosveld's disclosure may have appeared to him to suggest the claimed methods, it is only with Appellant's invention in hand that he was able to make such a combination, and the rejection is, therefore, an impermissible hindsight reconstruction of the claimed methods. *See In re Kotzab* 55 USPQ2d 1313, 1318 (Fed. Cir. 2000).

Thus, for the reasons of record, Appellants reiterate that no combination of teachings from Grosveld suggest the methods set forth in the claims on appeal, nor has the Examiner identified any motivation for one of skill in the art to combine the teachings the Examiner has selected to arrived at the claimed methods. Accordingly, a *prima facie* case of obviousness has not been and cannot be established based on Grosveld.

3. Intrinsic Evidence

The Examiner also cites case law in an attempt to support the assertion that the intrinsic evidence of the specification is always determinative of the meaning of a claim term. From the cited cases, the following conclusions appear to be drawn: (1) that the normal meaning of the term "library" in the art of molecular biology does not apply to the pending claims and (2) that Appellants' description of the term "library" on page 46 is not subject to any interpretation, either by other portions of the specification or by documents available at the time of filing. (Examiner's Answer, page 11). With regard to the second of the Examiner's conclusions, it is also maintained, seemingly on the grounds that the single sentence on page 46 must be viewed in isolation of the rest of the specification and state of the art, that this sentence somehow requires the interpretation that DNA libraries as claimed may include clones containing one or more copies of the same sequence. This mistaken interpretation has led the Examiner to reject, summarily and repeatedly, Appellant's argument that the normal and customary use of the term "library" in the field of molecular biology refers to collections of multiple, different fragments of DNA.

(a) *Normal meaning of the term in the art supercedes intrinsic evidence*

As an initial matter, Appellants traverse the Examiner's assertion that the specification is the primary source for determining the meaning of claim terms. To the contrary, the primary determinant of the meaning of a claim term is, and always has been, the normal meaning of the term in the art. *See*, for example, *Moba, B.V. et al. v. Diamond Automation, Inc.*, 66 USPQ2d 1429, 1433 (Fed. Cir. 2001):

> As a general rule, claim language is given the ordinary meaning of the words in the normal usage of the field of the invention. [Citing *Toro Co. v. White Consol. Indus.*, 199 F.3d 1295, 1299, 53 USPQ2d 1065, 1067 (Fed. Cir. 1999).]

Indeed, even one of the very cases cited by the Examiner, in the Examiner's Answer, cautions against ignoring the ordinary and customary meaning of a claim term:

> Consulting the written description and prosecution history as a threshold step in the claim construction process, before any effort is made to discern the ordinary and customary meanings attributed to the words themselves, invites a violation of our precedent counseling against importing limitations into the claims. *Texas Digital Systems, Inc. v. Telegenix, Inc.*, 308 F.3d 1193, 64 USPQ2d 1812, 1819 (Fed. Cir. 2002), *cert. denied*, 538 U.S. 1058 (2003)

In the pending case, the Office has ignored these cautions and instead has artificially broadened the claim term "library" by ignoring its ordinary and customary meaning, based on an isolated selection from the specification. In light of a record that clearly shows that the ordinary meaning of the term "library" in the art of molecular biology does not include a collection of identical molecules, the Examiner's interpretation of this claim term is improper. *See*, for example, Appendix B attached to the Appeal Brief dated September 30, 2004, in which a library is defined as "a large number of recombinant DNA clones, *each one of which contains a different segment of foreign DNA*." (emphasis added).

Thus, the Examiner has failed to recognize the normal meaning of the term to the skilled artisan and, as a result, has incorrectly construed the claim.

(b) *Appellants' definition is not contrary to normal and customary meaning of the term "library"*

Appellants also strongly dispute the Examiner's contention that the remarks relating to the term "library" on page 46 of the specification should be viewed, in isolation, as the sole definition of the term.. The specification can be used to define the meaning of a claim term if applicant, serving as his or her own lexicographer, has provided a new definition, or one that is different from the customary meaning of the term in the art. *See*, for example, *Brookhill Wilk 1, LLC v. Intuitive Surgical, Inc.*, 334 F.3d 1294, 1300, 67 USPQ2d 1132, 1136 (Fed. Cir. 2003):

> The presumption [of ordinary and customary meaning of a claim term] will be overcome where the patentee, acting as his or her own lexicographer, has clearly set forth a definition of the term different from its ordinary and customary meaning. [Citing *in re*

Paulsen, 30 F.3d 1475, 1480, 31 USPQ2d 1671, 1674 (Fed. Cir. 1994); *Intellical, Inc. v. Phonometrics, Inc.*, 952 F.2d 1384, 1387-88, 21 USPQ2d 1383, 1386 (Fed. Cir. 1992)]

In the pending case, the Examiner fastens upon Appellant's statement at page 46 of the specification to support an assertion that the term "library" has been re-defined by applicants. However, this statement[2] is in fact entirely consistent with the art-recognized meaning of the term "library." Moreover, numerous other portions of the specification use the term "library" in a manner consistent with its normal and customary meaning in the art of molecular biology (*i.e.*, a collection of different sequences), for example:

- page 28, lines 6-7: "Isolated polynucleotide *fragments* corresponding to *accessible regions* can be cloned and used to prepare *collections or libraries* of accessible regions." (emphases added, note use of the plural and note equivalence of the terms "collections" and "libraries")
- page 38, lines 21-23: ". . . the isolated *fragments* can be cloned to generate a library of regulatory *sequences*. The *nucleotide sequences* of the *members of the library* can be determined . . ." (emphases added, note uses of the plural)
- page 45, lines 7-9: "*Collections* of *accessible region sequences* from a particular cell can be cloned to generate a library, and the *nucleotide sequences* of the *members of the library* can be determined . . ." (emphases added, note use of plural)[3]
- page 47, lines 5-6: "The libraries formed can represent *accessible regions* for a particular cell type or cellular condition." (emphasis added, note use of plural)[4]
- page 47, lines 15-17: "Determination of the *nucleotide sequences* of the *members of a library* can be used to generate a database of *accessible sequences* specific to a particular cell type." (emphases added, note use of plural)

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[2] that a library is a pool of DNA fragments that have been propagated in a cloning vector
[3] Also presented in Appellants' Response dated May 18, 2004 at pp. 3-4.
[4] Also presented in Appellants' Response dated May 18, 2004 at page 3

Moreover, the working examples provided in the specification show libraries containing different sequences. *See* Example 15, pp. 112-116 and SEQ ID NOS. 10, 11 and 12.

Given that the specification makes clear throughout that the claimed libraries necessarily include "collections" of "accessible region sequences" and are libraries in which the "nucleotide sequences" of the "members of the library" can be determined, it is unclear to Appellants how the such libraries could, under any circumstances, consist of identical sequences, as asserted by the Examiner.

In sum, Appellants again note that, even if the normal and customary meaning of the term "library" in the art of molecular biology is disregarded, as the Examiner appears to have done, the intrinsic evidence of the specification is all that is necessary in order to properly construe the term "library" to refer to clones of multiple, different DNA sequences. Moreover, nothing in the specification indicates that Appellants intended a definition of library different from its normal and customary meaning in the art of molecular biology.

(c) *Intrinsic evidence not limited to specification*

Furthermore, the Examiner's insistence on the specification as the sole source of intrinsic evidence is misplaced. Rather, the intrinsic evidence, as pointed out in one of the cases cited on page 11 of the Examiner's Answer, includes **both** the specification and the file history:

> The intrinsic record includes the specification and the file history. [Citing *Masco Corp. v. United States*, 303 F3d 1316, 1324 (Fed. Cir. 2002)] *C.R. Bard, Inc. v. U.S. Surgical Corp.*, 73 USPQ2d 1011 (Fed. Cir. 2004) .

In the case at hand, the file history has made it abundantly clear that the claimed methods generate nucleic acid libraries composed of a collection of different nucleotide sequences. *See* Response dated December 17, 2003 (Resubmitted February 26, 2004) at pp. 6-8; Response dated May 18, 2004 at pp. 2-3 and Exhibits A and B attached to Response dated May 18, 2004; Response dated June 16, 2004 at p. 6 and Appeal Brief

dated September 30, 2004 at pp.12-14 and Appendices B and C attached to Appeal Brief.[5]

Thus, it is improper and incorrect for the Examiner to assert: (1) that the intrinsic evidence supports his proposed definition of the term "library" to include a clone and (2) that Grosveld teaches a method which prepares libraries as claimed. *Examiner's Answer*, page 13.

(d) *Claim term must be taken in context*

Moreover, the meaning of a claim term must be taken within the context of the claim itself:

> . . . the context of the surrounding words of the claim also must be considered in determining the ordinary and customary meaning of those terms. [Citing *Hockerson-Halberstadt, Inc. v. Converse, Inc.*, 183 F.3d 1369, 1374, 51 USPQ2d 1518, 1522 (Fed. Cir. 1999)] *Brookhill Wilk 1, LLC v. Intuitive Surgical, Inc.*, 334 F.3d 1294, 1300, 67 USPQ2d 1132, 1136 (Fed. Cir. 2003)
>
> The words used in the claims must be considered in context and are examined through the viewing glass of a person skilled in the art. *Ferguson Beauregard/Logic Controls, Division of Dover Resources, Inc. v. Delaware Capital Formation, Inc.*, 69 USPQ2d 1001, 1009 (Fed. Cir. 2003)
>
> Moreover, as this court has repeatedly counseled, the best indicator of claim meaning is its usage in context as understood by one of skill in the art at the time of invention. [Citing *Markman*, 52 F.3d at 986.] *Moba, B.V. et al. v. Diamond Automation, Inc.*, 66 USPQ2d 1429, 1435 (Fed. Cir. 2001)

In the present case, the claims recite contacting a cell nucleus (comprising cellular chromatin) with an enzyme (claim 123) or a probe (claim143) which reacts with accessible regions. It is well-known to those of skill in the art that cellular chromatin contains a plurality of accessible regions. *See*, for example, Gross *et al.* (1988) *Ann. Rev. Biochem.* **57**:159-197, wherein the following statements are made:

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[5] For that matter, the specification also discloses a library containing multiple different nucleic acid sequences. *See* Example 15 at pp. 112-116, including SEQ ID Nos. 10, 11 and 12.

> These accessible regions are operationally defined by their pronounced sensitivity to nuclease cleavage or chemical modification . . .
>
> Hypersensitive sites generally represent a minor (ca. 1%), but highly selective fraction of the genome.
>
> . . . the sites themselves most often encompass a unit length close to that of the nucleosomal repeat (ca. 200 bp) or several multiples thereof . . .
> (All quotes taken from Gross *et al.* p.160)

Applying the information provided by Gross to a mammalian genome of ca. 3 x 10^9 base pairs, 1% of such a genome would contain 3 x 10^7 base pairs. If each accessible region were 200 nucleotides in length, a total of 3 x 10^7/2 x 10^2 = 1.5 x 10^5 accessible regions are present. Thus, in an average mammalian genome, 150,001 fragments would be produced by an enzyme that cleaves accessible regions. These 150,001 fragments are then subjected to further fragmentation. *See* claim 123, step (c); claim 143, step (b). It should therefore be clear that, in the context of the claims, one of skill in the art of molecular biology would realize that over 150,001 fragments are produced when the claimed method is practiced with a typical mammalian genome. It should also be clear that, even for smaller genomes and/or larger accessible regions, multiple fragments will be produced.

Consequently, it is not reasonable to assert, as the Examiner has done, that the practice of the claimed methods could generate a library consisting solely of multiple copies of the same sequence.

(e) *Examiner's proposed definition of "library" is repugnant to its normal meaning in the art*

Finally, the definition of "library" propounded by the Examiner is not only contrary to the normal and customary meaning of the term in the art, it is actually repugnant to that meaning. One or more nucleic acid sequences propagated in a vector molecule is denoted a "clone" in the art of molecular biology; a collection of clones

having different inserts[6] is denoted a "library." *See*, for example, Appendix B attached to the Appeal Brief dated September 30, 2004, in which a library is defined as "a large number of recombinant DNA clones, each one of which contains a different segment of foreign DNA." Immeasurable confusion would rage in the field of molecular biology if these two terms were mistakenly intermixed, as has been done by the Examiner. To refer to a single clone as a library, based on the presence of multiple identical copies of the clone in a sample, as the Examiner has done, is akin to identifying a building containing one million copies of the same book as a library.

Thus, the Examiner has improperly and incorrectly re-defined the term "library" to the point that the term has been removed so far from its normal and customary meaning as to be essentially meaningless.

(f) *The record as a whole supports Appellants' claim construction and refutes Examiner's*

Appellants have shown that the normal and customary meaning of the term "library" in the art of molecular biology is consistent with their use of that term in the specification and claims. They have further shown that a proper consideration of the intrinsic evidence, including both the specification and the file history, does not reveal any attempt to contradict or somehow re-define this normal and customary meaning. The various art definitions provided by Appellants, which have been dismissed by the Examiner, constitute additional intrinsic evidence supporting Appellants' interpretation of the claim language, inasmuch as they are of record and are part of the file history. These art-recognized definitions are not, in any way, inconsistent with the specification's definition of the term "library." Rather, they entirely supports Appellants' interpretation that a library contains clones of different DNA sequences.

Therefore, when properly interpreted, the claims are drawn to methods of making DNA libraries in which the sequences making up the library are different. Furthermore,

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[6] Such as might be obtained, for example, by: (1) cleaving genomic DNA with a restriction enzyme and cloning the resulting collection of fragments to generate what is known in the art as a "genomic library" or (2) reverse transcribing a population of mRNA molecules and cloning the resultant pool of DNA molecules to generate what is known in the art as a "cDNA library"

under this accurate interpretation, it is clear that the claims are nonobvious over Grosveld.

4. Preamble

After asserting on page 10 that the only issue relates to intrinsic evidence, the Examiner then maintains, on page 13, that a "second issue is whether the preamble limitation of 'library' constitutes a structural limitation on the method claim." (Examiner's Answer, page 13).

For the reasons noted above, the steps of the claims on appeal are different from Grosveld whether or not the term "library" is included in the preamble.

Appellants also note that they were not allowed to put language into the claims positively reciting that the plurality of sequences of the clones are different from each other. *See,* Supplemental Amendment filed June 16, 2004. Inasmuch as the Examiner was on record as asserting that the claimed methods included both libraries containing multiple sequences and libraries containing a single sequence (Office Action of September 29, 2003 at page 2; Office Action of April 13, 2004 at pp. 9-10; Advisory Action of June 2, 2004), it was believed that this amendment represented a narrowing of the claim and would simplify issues for appeal.

Despite the Examiner's express encouragement to submit this supplemental amendment, and the clear support in the specification as filed (*e.g.,* working examples), entry thereof was refused. *See* Advisory Action dated June 29, 2004. Appellant submits that the Examiner's refusal to enter the amendments, especially after encouraging Appellant to make them, has resulted in unnecessary delays and expense, particularly in the form of this Appeal process. Appellant's good faith attempt to avoid the Appeal process by amending the claims to make explicit what was originally implicit was improperly denied by the Examiner and would obviate any remaining issues.

In support of the refusal to enter the amendments, the Advisory Action simply stated "The arguments are not considered since they relate to the amendment which was not entered." Accordingly, Appellants remain unaware of why the amendments initially encouraged by the Examiner were subsequently not entered.

5. Library Motivation

Finally, the Examiner's Answer asserts again that Grosveld teaches the "manipulative steps" of the claims and points to a paper regarding cosmid libraries cited at col. 13, lines 56-58 of Grosveld. (Examiner's Answer, pages 14-15). The Examiner's Answer also cited MPEP 2144 for the proposition that Grosveld need not contain the same motivation as the specification. *Id.*

The citation to MPEP 2144 cannot change the fact that Grosveld does not teach or suggest either the "manipulative steps" or the libraries themselves. For the reasons detailed above, Grosveld does not teach the methods of the claims on appeal and contains no motivation to alter the methods he does disclose to arrive at the claimed subject matter.

Moreover, the rule that the motivation need not be the same is irrelevant in this case. What is relevant is the well-settled case law clearly and consistently holding that there is no motivation to alter a reference where the proposed motivation would destroy the intended function of the reference, as set forth, for example in *In re Fritch* 23 USPQ2d 1780, 1783, n.12 (Fed. Cir. 1992) and *In re Ratti* 123 USPQ 349, 352 (CCPA 1979):

> A proposed modification [is] inappropriate for an obviousness inquiry when the modification renders[s] the prior art reference inoperable for its intended purpose.
>
> [I]t would require a substantial reconstruction and redesign of the elements shown in [a cited reference] as well as a change in the basic principles under which [that reference's] construction was designed to operate.

In sum, if the Office's efforts to attain the claimed subject cause the reference to become inoperable or destroy its intended function, then the requisite motivation to make the modification would not have existed.

Here, both the Grosveld references are directed to cloning of hypersite-encompassing fragments in order to prepare constructs used for gene integration. *See, e.g.,* Abstract and Examiner's Answer page 14, describing cosmids of Grosveld paper.

Thus, the intended function of Grosveld is to make constructs in which a coding sequence (gene) is linked to the fragments encompassing the hypersites. Modifying Grosveld as suggested by the Examiner to result in a library as claimed would completely destroy the sole intended function of Grosveld's constructs, at the least because Grosveld's constructs would not contain a coding sequence.

Thus, no matter what the Examiner alleges as the motivation to alter Grosveld to arrive at the claimed subject matter, such altering would: (1) result in a completely different process than that claimed and (2) fundamentally destroy the function of Grosveld's constructs. Accordingly, there is no motivation that can be used to justify the rejection and the Examiner's decision should be reversed.

CONCLUSION

For the reasons stated above, Appellant respectfully submits that the pending claims define subject matter that is nonobvious over the art cited by the Examiner.

Accordingly, Appellants request that the rejections of the claims on appeal be reversed, and that the application be remanded to the Examiner so that the appealed claims can proceed to allowance.

Respectfully submitted,

Date: January 27, 2005

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NUCLEASE HYPERSENSITIVE SITES IN CHROMATIN

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PERSPECTIVES AND SUMMARY

Eukaryotic chromosomes participate in transcription, replication, meiotic and mitotic condensation, pairing, recombination, and segregation. These processes occur through specific interactions between nuclear proteins and chromosomal DNA sequences. Recognition of specific DNA sequences by proteins requires accessibility. In chromatin, nucleosome-free regions known as *nuclease hypersensitive sites* are believed to represent the "open windows" that allow enhanced access of crucial resident *cis*-acting DNA sequences to *trans*-acting factors (see Refs. 1–7a for earlier reviews). These accessible regions are operationally defined by their pronounced sensitivity to nuclease cleavage or chemical modification, and are typically two orders of magnitude more sensitive than other regions in bulk chromatin. Hypersensitive sites generally represent a minor (ca. 1%), but highly selective fraction of the genome. These local regions should not be confused with long segments of the chromatin fiber extending over many kilobases (kb), associated with potentially active or actively transcribed genes, that exhibit a heightened general sensitivity to nucleases of perhaps an order of magnitude (8).

Nuclease hypersensitive sites were first discovered in studies of SV40 viral chromatin structure in 1978, independently by Varshavsky and coworkers (9) and by Scott & Wigmore (10). Their presence in cellular chromatin was first recognized by Wu & Elgin in 1979 (11). We now know that these sites are fundamental elements in biology as they are ubiquitous among the eukaryotes, being found in the cellular chromatin of plants, animals, and fungi as well as within viral or episomal genomes (see Table 2).

The method most commonly used to map nuclease hypersensitive sites is DNase I digestion of nuclei followed by indirect end-labeling of the resulting purified double-stranded DNA (14). However, the resolution of this method has been generally overestimated, and these regions are now being elucidated by a wide variety of other enzymatic and chemical probes in conjunction with alternative mapping procedures that yield single-nucleotide resolution (see Table 1). While the sites themselves most often encompass a unit length close to that of the nucleosomal repeat (ca. 200 bp), or several multiples thereof, the fine structure of any given site often exhibits multiple "hot" and "cold" spots that vary depending upon the cleavage reagent used (see Figure 1). Sites are hypersensitive because of the absence of a canonical nucleosome, but contain cold spots (footprints) that reflect the presence of bound *trans*-acting factors.

Within a given cell type, DNase I–hypersensitive sites fall into two major categories: *constitutive* and *inducible*. Constitutive sites are often present in promoter regions of genes "poised" for transcriptional induction; their presence precedes transcriptional activation and is independent of gene expression. Inducible sites also can appear prior to transcription and often persist

long after removal of the inducing agent and/or continued transcription. Sites that differ between cell types are termed *tissue-specific*, while tissue-specific sites that appear transiently during embryogenesis are called *developmental* (see Figure 2).

A wide variety of functional sequences are associated with hypersensitive sites. For example, in *Saccharomyces cerevisiae*, hypersensitive sites occur around centromeres, silencers, recombination sequences, replication origins, upstream activation sequences (UASs), promoter elements, and presumptive transcription terminators (see Table 2). Thus, it seems clear that these sites are nearly always associated with *cis*-acting DNA sequences.

Several classes of nuclear proteins have been found associated with a subset of hypersensitive sites, including topoisomerases I and II, RNA polymerase II, and transcription factors. The available evidence suggests that histones are absent from these regions. The proteins associated with most sites, however, have not yet been identified.

The mechanisms leading to the formation, maintenance, and propagation of hypersensitive sites are poorly understood and represent challenging questions. Because of the diversity of biological functions associated with such sites, it seems probable that several different mechanisms may be involved in these processes. Interaction of the resident DNA sequences with *trans*-acting factors is one likely mechanism for hypersensitive site formation, but the generation of these regions can also be regulated by sequences at far distances. DNA methylation, looping, sequence composition, alternative conformations, and/or torsional stress may play roles in some of these processes and be induced by localized protein binding. Often nucleosomes are positioned specifically along the DNA sequences residing adjacent to or between hypersensitive sites (see Figure 1D). In many cases this positioning has been shown to occur by an "active" mechanism that depends on the underlying DNA sequence and not on the presence of adjacent hypersensitive sites. While DNA bendability and sequences localized close to the dyad axis of the nucleosome seem to determine nucleosome positioning, whether alternative nucleosome phasing frames might regulate hypersensitive site formation poses an interesting question. Clearly, knowledge of these basic principles should provide insight into the molecular bases of gene regulation and have important future applications in engineering optimally regulated *trans*-genes for scientific, therapeutic, and commercial benefit.

EXPERIMENTAL DETECTION OF HYPERSENSITIVE SITES

DNA Cleavage and Base Modification Techniques

Table 1 lists a variety of probes that are available to cleave DNA or modify bases within hypersensitive sites. As can be seen, these reagents exhibit a

broad spectrum of specificities, and many are capable of acting directly on living cells. It should be appreciated that exposure of cells or nuclei to a variety of compounds (e.g. detergents, polyamines, oxidizing agents, and metal chelators) prior to or during the cleavage or modification steps may affect the results. When considering which agent to use for detecting hypersensitive sites, the following caveats should be borne in mind: (*a*) The probe should be highly selective for these sites. By definition, nuclease hypersensitive sites are the DNA regions most susceptible to attack in chromatin; however, internucleosomal and, to a lesser extent, intranucleosomal DNA are normally also attacked but at far lower rates. (*b*) Agents with high sequence specificity, such as micrococcal nuclease and restriction enzymes, should generally only be used for detecting bound factors. (*c*) Probes capable of penetrating living cells minimize the possibility of protein rearrangement that may occur upon cell lysis and/or subcellular fractionation. (*d*) Since DNA supercoiling can induce structural perturbations over long distances, agents that directly cleave DNA have the potential to alter such supercoil-dependent structures (55, 56). (*e*) Reagents that intercalate induce DNA unwinding and diminish negative supercoiling, which can lead to a similar outcome as in (*d*). In addition, intercalation causes protein displacement (57–59). (*f*) Alkylating agents, which modify bases so that DNA cleavage can be achieved later, also react with certain amino acid side chains in proteins and hence can lead to secondary effects. (*g*) Cleavage may not even be the result of the added probe, since endogenous nucleases are sometimes activated upon exposure of cells to drugs (60) or of nuclei to various divalent metals (61–64). (*h*) Topoisomerase I and II cleavage sites can differ depending on the methods used to induce the protein-linked DNA breaks (65, 66).

While it is beyond the scope of this article to discuss the varied applications, advantages, and disadvantages of each of the agents listed in Table 1, it seems clear that there is no substitute for an experimental approach that uses several probes with alternate modes of action. Nevertheless, for general applications DNase I is the enzyme of choice because of its high selectivity for nucleosome-free regions and relatively low DNA sequence specificity (67, 68). In addition, UV light (69, 69a, 175, 198) and/or dimethylsulfate (DMS) (70–74, 165, 166) treatment of living cells have been very effective in probing hypersensitive site substructure at nucleotide-level resolution. DMS offers the added benefit of being a groove-specific probe: it modifies guanines in the major groove (at the N7 position) and adenines in the minor groove (at the N3 position) (161). Finally, methidiumpropyl-EDTA (MPE) cleavage has yielded striking results in several studies (75, 76, 169, 191, 193), but since MPE intercalates, it may be superceded by the related, but nonintercalative, iron(II)-EDTA technique for some applications [see (*e*) above] (77, 78, 194, 197).

Mapping the Positions of Hypersensitive Sites

INDIRECT END-LABELING Traditionally the positions of hypersensitive sites have been mapped by a moderate-resolution indirect end-labeling technique, developed independently by Wu (14) and Nedospasov & Georgiev (176). In this procedure, cleavage of nuclear chromatin is followed by DNA purification, restriction endonuclease digestion, electrophoretic separation of the resulting DNA fragments on nondenaturing agarose gels, Southern transfer, and hybridization with a radiolabeled recombinant DNA probe chosen to abut the restriction site. This effectively labels a nested set of daughter fragments defined at one end by the restriction site and at the other end by the "in vivo" cutting site(s); these cleavage points are then mapped by determining subfragment sizes. This technique, however, has several limitations that can lead to inaccuracies in positioning hypersensitive sites within DNA sequences: (*a*) the size of large fragments (>1 kb) can be determined with an accuracy of generally no better than ±50 bp; (*b*) most workers do not mix size standards with their samples to control for lane-to-lane variation in electrophoretic mobility; (*c*) the fragments most likely will have protruding single-stranded ends, and internal nicks or single-stranded gaps, since most cleavage reagents (e.g. DNase I) attack each DNA strand separately. Such fragments may migrate anomalously during electrophoresis. In general, the high-resolution techniques described in the next section are free of these limitations.

ELECTRON MICROSCOPY Obviously, electron microscopic techniques are limited in resolution and require the means to identify and/or enrich the desired molecules. This approach, however, has been very successful in studies of SV40 minichromosomes, since the nuclease hypersensitive region can be shown to appear nucleosome-free (186–189).

Mapping the Fine Structure of Hypersensitive Sites

GENOMIC FOOTPRINTING This procedure is the current method of choice for mapping at nucleotide resolution "hot" and "cold" spots within the hypersensitive sites of complex cellular genomes (69–74, 165, 166, 178). The technique is based on the genomic sequencing procedure pioneered by Church & Gilbert (156) and utilizes electrophoretic transfer of the restricted DNA subfragments from an acrylamide sequencing gel to a nylon membrane, followed by UV crosslinking to covalently link the DNA fragments, and hybridization with strand-specific probes that abut the restriction site.

PRIMER EXTENSION This rapid method employs short radiolabeled oligonucleotide primers that are annealed to denatured DNA fragments, and then

Table 1 Properties of agents useful for probing hypersensitive sites

DNA cleavage or base modifying agent	Action on living cells	Substrate specificities	Examples of genes studied	References
I. Nucleases				
Bal-31	–	Duplex fragment ends or internal regions with single-stranded or non–B-form character	*Drosophila* hsp23, hsp26, hsp28; SV40; potential Z-forming sequences; human dihydrofolate reductase	79–81, 201
DNase I	–	Minor groove (12 Å width optimal); ApN, TpN, NpA, NpT dinucleotide sequences	Chicken α- and β-globin; chicken lysozyme; *Drosophila* hsp70, hsp82; yeast *HSP82*	8, 14, 46, 67, 68, 82–90, 177, 178, 239
DNase II	–	Stacked single-strand	*Tetrahymena* ribosomal RNA; chicken β-globin	68, 81, 82, 85, 91–95, 192, 239
Endogenous nucleases	±	Enzyme dependent	SV40; chicken ovalbumin; human c-Ki-*ras;* *Dictyostelium* ribosomal RNA and cysteine proteinase	10, 60–64
Exonuclease III	–	Double-stranded DNA ends free of tightly bound proteins	*Drosophila* hsp70, hsp82; mouse mammary tumor virus LTR	96–102
Lambda exonuclease	–	Double-stranded DNA ends free of tightly bound proteins	Reconstituted chromatin	437
Micrococcal nuclease	–	dA·dT-rich duplexes, single-stranded preferred	*Xenopus* 5S RNA; SV40; *Drosophila* histone; yeast *HSP82*	46, 67, 68, 82, 85, 103–111, 176
Mung bean nuclease	–	Single-stranded and dA·dT-rich non–B-form duplexes	*Drosophila* hsp23, hsp26, hsp28; SV40; yeast *ARS* and *LEU2*	79, 112, 113, 384, 471
Neurospora crassa nuclease	–	Single-stranded and non–B-form duplexes	Chicken β-globin; *Drosophila* hsp70	114, 115, 212
Restriction endonucleases	–	Sequence specific	SV40; HSV thymidine kinase; MMTV LTR; chicken β-globin; *Drosophila* hsp70, hsp82; mouse Igκ; yeast *PHO5*	9, 34, 41, 47, 80, 98, 99, 102, 116–119, 239, 291
S1 nuclease	–	Single-stranded and non–B-form duplexes	*Drosophila* heat shock loci; chicken β-globin; chicken collagen; adenovirus late	79, 114, 115, 120–123, 155, 199, 212, 314, 346, 384

Topoisomerase I	±	Supercoiled DNA preferred; the consensus sequence: $A^A_GACTTAGA^A_GAAA^{AA}_{TTT}$	*Tetrahymena* ribosomal RNA; SV40; *Drosophila* heat-shock loci; rat tyrosine amino transferase	65, 124–132
Topoisomerase II	±	The consensus sequence: $GTN^A_TA^C_TATTNATNN^A_G$	SV40; *Drosophila* heat-shock and histone loci	66, 124, 125, 133–139
II. Chemical/physical agents				
Bleomycin	+	Sequence-selective cleavage	Retroviral hybrid; *Drosophila* histone; chicken β-globin	56, 140–142, 148
Bromoacetaldehyde	+	Modifies unpaired bases	Chicken β-globin; poly(dG)-poly(dC)	143, 144
Chloracetaldehyde	+	Modifies unpaired bases	Potential Z-forming sequences	145
Cobalt chiral complex	?	Induces photoactivatable single-strand breaks in underwound duplexes and non–B-form DNA	SV40	146, 147, 196
Copper phenanthroline	?	Intercalates and cleaves dA·dT-rich regions in duplex DNA preferentially	*Drosophila* histone and heat-shock loci	68, 148–150
Diethyl pyrocarbonate	+	Modifies purines in *syn* conformation (e.g. those in Z-form DNA)	Chicken β-globin, antibody-Z DNA complexes	151–154
Dimethyl sulfate	+	Modifies N7 of G, N3 of A in duplexes, plus N1 of A and N3 of C in single-stranded DNA	Mouse immunoglobulin μ; human histone H4 and κ immunoglobulin; *E. coli lac* operator; Maize *Adh1;* rat tyrosine aminotransferase; yeast UAS_{GAL}	70–74, 156–166, 200
Iron(II)-EDTA	?	Hydroxyl radical mediated DNA cleavage	Lambda operator O_R1; Herpes thymidine kinase	77, 78, 197
Methidiumpropyl-EDTA	?	Intercalates and cleaves duplex DNA with generated hydroxyl radicals	*Drosophila* 5S RNA and heat-shock loci; mouse β globin; *Oxytricha* telomeres	75, 76, 167–169, 191, 193, 195
Neocarzinostatin	+	Sequence-selective cleavage	Retroviral hybrid	142
Psoralen	+	Photoactivable duplex interstrand crosslinker; intercalator	SV40; *Dictyostelium* ribosomal RNA	170–174
Ultraviolet light	+	Induces cyclobutyl pyrimidine dimers and 6-4 pyrimidine adducts	*E. coli lac* operator; yeast *GAL1-GAL10*	69, 69a, 175, 198

extended with either DNA polymerase (179, 180) or reverse transcriptase (181) to the site of cleavage. The products are then directly analyzed on sequencing gels. This technique is the method of choice for the analysis at single-nucleotide resolution of small genomes, such as those found in viruses (180), prokaryotes (179), and yeast (181), but procedures that enrich for specific DNA sequences may allow future application to complex genomes.

DIRECT END-LABELING Garguilo & Worcel (108) developed a technique in which plasmids are end-labeled within a unique restriction site and recircularized prior to microinjection into amphibian oocytes for chromatin assembly. Subsequent to in vivo cleavage by microinjected enzymes, purified DNA is relinearized and directly analyzed on a sequencing gel. Endogenous episomes that are probed in vivo with an alkylating agent may also be end-labeled after the modified DNA is purified (182).

SINGLE-STRAND–SPECIFIC NUCLEASE MAPPING Felsenfeld and coworkers (183, 295) and Weintraub (184) have independently devised a modification of the S1 mapping procedure (185), in which an end-labeled or uniformly labeled single-stranded probe is annealed to the cleaved sequence of interest. Complexes treated with single-strand–specific nucleases are then resolved on native (183) or denaturing (184) gels. The accuracy of this method is limited by the precision of action of the nuclease (±2 bp).

PRIMER LIGATION Kuo and coworkers (190) have developed a technique that depends upon ligating a labeled primer to the sequence of interest at an end defined by a restriction site. This is achieved by employing a single-stranded recombinant DNA as a "scaffold" to align by hybridization the labeled primer with the fragment. This method has theoretical advantages but appears to require additional development.

EXONUCLEASE BARRIERS Wu (98) has introduced a technique to map the boundaries of protein-binding sites within nuclease hypersensitive regions. Nuclear chromatin is digested with a restriction endonuclease and then with exonuclease III, and the barriers to exonuclease nibbling are mapped by indirect end-labeling of S1 nuclease–treated purified DNA. The procedure has been highly successful in a variety of studies (98–102, 201, 318). Bal-31 and lambda exonuclease variations of this procedure have also been employed (80, 201, 437).

MORPHOLOGICAL FEATURES OF HYPERSENSITIVE SITES

Individual hypersensitive sites have a characteristic morphology that is experimentally reproducible under a standard set of conditions. However, the

appearance of a given site will often be distinct from that of another and, furthermore, will vary depending upon the nuclease or chemical probe used (14, 76, 148, 206). The bracketed regions shown in Figure 1 denote four different constitutive DNase I–hypersensitive sites found in *Saccharomyces cerevisiae* that illustrate some features of these elements that are ubiquitous among eukaryotes (8, 12–23, 27–34, 45, 46). The degree of DNase I hypersensitivity within each of these sites varies compared to that of the corresponding flanking sequences and follows the order A > B > C > D. Micrococcal nuclease, on the other hand, often cleaves strongly at the borders of DNase I–hypersensitive regions (e.g. Figure 1B), and this sometimes correlates with additional cleavages at nucleosomal periodicities along adjacent sequences (Figure 1A, 1D), suggesting the presence of flanking sequence-positioned (phased) nucleosomes (see below). The minimum unit size of hypersensitive sites corresponds to the nucleosome repeat length, implying that sites may acquire a nucleosomal structure under certain environmental conditions, but sites as large as 1.3 kb have been reported (207). It is clear that hypersensitive sites contain multiple "hot" and "cold" spots of nuclease cutting; these are readily observable at the level of resolution shown in Figure

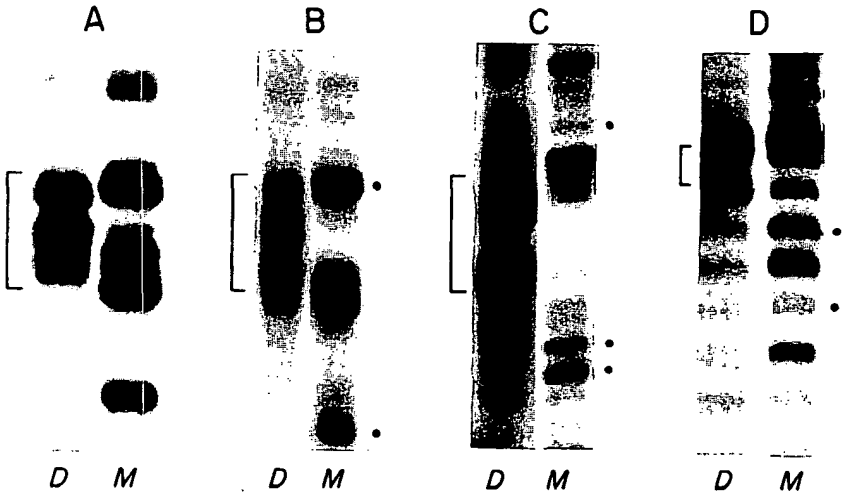


Figure 1 Montage of nuclease hypersensitive sites within the *HSP82* locus of *Saccharomyces cerevisiae*. Indirect end-labeling of nuclease hypersensitive sites after DNase I (*D*) or micrococcal nuclease (*M*) digestion of nuclei. DNA was separated by electrophoresis, from top to bottom, on nondenaturing agarose gels. The brackets represent 170 bp, the average nucleosome repeat length and hypersensitive site unit length in yeast (46, 205). All patterns depicted are chromatin-specific except for the bands indicated by closed circles, which are also found in control digests of naked DNA. Panels A, B, C, and D represent nuclease hypersensitive regions centered at +3350, -1820, -170, and -4500 relative to the *HSP82* trancription start site, respectively. [C. Szent-Gyorgyi, W. T. Garrard, unpublished results (see Refs. 45, 46)].

1. Such substructure has been demonstrated to be due to the presence of bound *trans*-acting factors in other systems (69, 69a, 72, 76, 98, 99, 208, 295). Obviously, nucleotide resolution throughout each strand of a hypersensitive site is necessary to completely describe the morphology of any such region.

BIOLOGY OF HYPERSENSITIVE SITES

Constitutive, Inducible, Developmental, and Tissue-Specific

Constitutive hypersensitive sites are usually found in the promoter regions of genes poised for transcriptional activation, such as the heat-shock inducible genes of *Drosophila* (14, 48, 76, 79, 149, 209–213), the mouse metallothionein gene (214), or certain inducible genes in *Saccharomyces cerevisiae* (26, 46). Such sites are also found within promoters of housekeeping genes that are expressed at low basal rates (15, 201, 215–219) (see Table 2 for a complete listing of sites associated with promoters and other functional sequences). While the presence of constitutive sites is independent of gene expression, *inducible* sites are most often (but not always, see Refs. 49, 50, 220) associated with concomitant transcriptional activation of the linked gene. Such sites are generally associated with genes regulated by steroid hormones (31, 50, 51, 73, 90, 102, 142, 191, 206, 209, 222–227, 229, 279), but a lipopolysaccharide-inducible hypersensitive site is found in the κ immunoglobulin gene enhancer of mouse pre–B-cells (230, 231), and light- and oxygen-tension-inducible hypersensitive sites exist in the sequences flanking responsive plant genes (12, 13). Inducible sites have also been found associated with expression of fungal genes (22, 23a, 41, 232). *Developmental* sites appear transiently within the promoters of embryonic and fetal chicken (8, 89, 233) and human (16, 233–238) globin genes concomitant with their transcription; these sites are expression-dependent. Differentiation-dependent sites have also been found surrounding the chicken histone H5 gene (240), the mouse skeletal muscle acetylcholine receptor gene (241), a stage-specific gene in *Dictyostelium* (22), and *Drosophila* glue protein genes (242–247). Finally, it appears to be a general rule that any gene that will be, is, or has been expressed in a specific cell type will have linked *tissue-specific* hypersensitive site(s) (8, 17, 18, 75, 89, 224–227, 233–240, 248–252).

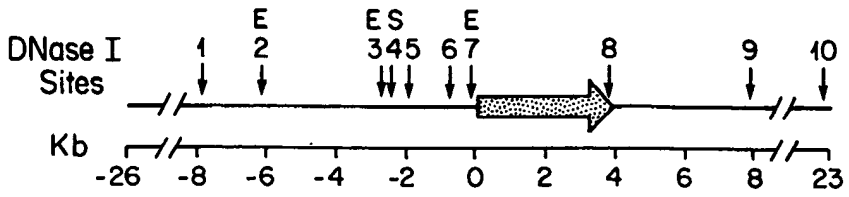
The chicken lysozyme gene locus is perhaps the best-studied example with respect to the biology of DNase I–hypersensitive sites. Although the transcription unit itself is only about 4 kb long, in oviduct nuclei the gene is embedded in the center of a 24-kb domain that is preferentially sensitive to nuclease (227). Figure 2 summarizes the studies of Igo-Kemenes, Sippel, and coworkers that have mapped every DNase I–hypersensitive site residing within 50 kb of this locus in nuclei from a variety of tissues and cell types (90,

224–227). Among the 10 hypersensitive sites identified, none is localized near the borders of the nuclease-sensitive domain, which reside at about -12 kb and +12 kb (227). The different sites can be classified into specific categories. Site 8 is the only one that is non–tissue-specific. Sites 3 and 6 are macrophage-specific, while Site 5 is specific to the oviduct and is hormone-inducible. Sites 2 and 7 are linked to the transcriptionally poised (potentially expressible) gene state. Interestingly, these correspond to transcriptional enhancers (253). Site 3 also corresponds to an enhancer (254), while Site 4 maps at a silencer sequence (255). Although the roles of sequences associated with the other sites are less certain, it seems clear that virtually all of the hypersensitive sites associated with the lysozyme gene locus exhibit biologically interesting patterns that directly correlate with function.

FUNCTIONAL SEQUENCES ASSOCIATED WITH HYPERSENSITIVE SITES

Enhancers, Silencers, Upstream Activation Sequences, Promoters, Terminators, Replication Origins, Topoisomerase Sites, Recombination Loci, Centromeres, and Telomeres

In essentially every case that has been adequately studied it is striking that hypersensitive sites are associated with sequences that function in processes such as transcription, replication, recombination, or chromosome segregation. Table 2 lists the hypersensitive sequences for which a function has been demonstrated or strongly implied. As is evident, the vast majority thus far described are gene-associated and appear to play a functional role in determining the position and frequency of transcriptional initiation and termination. It is of particular significance that in many instances the sites themselves were discovered years before any function was recognized for the resident sequences, especially in the case of transcriptional enhancers. In fact, it is now possible to generalize that if a *cis*-acting sequence is working to affect a chromosomal process, it will be associated with a hypersensitive site. For example, in mammals the promoter regions of genes on the active X-chromosome possess DNase I hypersensitive sites, while their allelic partners residing on the inactive X-chromosome lack such sites (320, 321). Similarly, the promoter of the histone H4 gene is organized into a DNase I–hypersensitive site in the transcriptionally active macronucleus of *Tetrahymena*, but the same sequence in the same cell is not hypersensitive in the transcriptionally inactive micronucleus (307). On the other hand, not all promoters that are hypersensitive are necessarily actively engaged in priming transcription. For example, 5'-hypersensitive sites can be experimentally induced in the absence of transcriptional activation of the chicken adult globin genes (49). Hence, hypersensitivity is necessary, but not sufficient for the



Tissue or Cell Type		Hypersensitive Site Number										Functional State of Gene	Transcription
		1	2	3	4	5	6	7	8	9	10		
Immature Oviduct	+DES	+	+		+	+		+	+	+	+	Induced	Yes
	−DES	+	+		+			+	+	+	+	Deinduced	No
Mature Oviduct		+	+		+	+		+	+	+	+	Active	Yes
Macrophage		+	+	+			+	+	+		+	Constitutive	Yes
Liver, Kidney, Brain, Embryo		+			+				+	+	+	Inactive	No
Erythrocyte									+			Dormant	No

Figure 2 Biology of DNase I hypersensitive sites within the chicken lysozyme gene locus. The transcription unit is depicted by the horizontal arrow. Different DNase I hypersensitive sites are indicated by the 10 numbered vertical arrows. Some correspond to transcriptional enhancer (E) and silencer (S) sequences. As indicated for the corresponding tissue or cell type, a plus sign means that the site is present, while a blank means that the site is absent. DES, diethylstilbestrol. Summary of work published in Refs. 90, 224–228, 253–255.

underlying DNA sequence to exert its function. Hypersensitivity reflects but one of several steps in the pathway of committing a *cis*-acting sequence to be functionally active in chromatin. While the functions of the sequences residing within many hypersensitive sites still remain to be established (e.g. see Figure 2), it would seem that it is only a matter of time before this is accomplished.

PROTEINS ASSOCIATED WITH HYPERSENSITIVE SITES

Histones

Although it has generally been assumed that nuclease hypersensitive sites lack histones because of their marked accessibility relative to nucleosomal DNA, only a few experiments have directly addressed this question. In an early study, Felsenfeld and coworkers (239) showed that the 5'-hypersensitive site of the chicken β-globin gene could be excised as a 115-bp

Table 2 Functional sequences associated with hypersensitive sites[a]

I. Transcriptional elements

A. Enhancers

1. Viral

Avian: endogenous *ev*-3 (29), leukosis (217), Rous sarcoma (27).
Human: cytomegalovirus (30).
Mouse: mammary tumor (31, 102, 142).
Polyoma: (33, 275, 276).
SV40: (9, 10, 34, 54, 80, 119, 186–189, 204, 257–274).

2. Cellular

Chicken: β-globin (277, 278), ovalbumin (206, 279, 280), lysozyme (90, 224–227, 253, 254), vitellogenin (50, 51, 281).
Drosophila: Sgs-4 (242–244, 246, 247).
Human: β-globin (233, 234, 282), ϵ-globin[b] (234–236), Igκ (74), IgH (283), c-Ki-*ras*[b] (63).
Mouse: Igκ (230, 231, 284–286), IgH (70, 71, 221, 256).
Rat: elastase I (252), serum albumin (248, 249), tyrosine aminotransferase (73, 222, 223).

B. Silencers

Chicken: lysozyme (90, 224–227, 255), ovalbumin (206, 279, 280).
Saccharomyces cerevisiae: HMR-E and *HML*-E regions (25, 287, 288), *PHO5* (40, 41, 47, 232, 290).

C. Upstream activation sequences

Saccharomyces cerevisiae: GAL1–GAL10 (38, 39, 42, 69, 69a, 72, 289), *PHO5* (40, 41, 47, 232, 290).

D. Promoters

1. Viral

Viral enhancer listings above plus:
Adenovirus: major late (184).
Herpes Simplex: thymidine kinase (291).

2. Cellular

Chicken: α-globin-cluster (49, 89, 120, 220, 296), β-globin-cluster (8, 49, 52, 60, 95, 115, 120, 143, 183, 220, 233, 239, 295), collagen (296), c-*myc* (217), histone H5 (241), lysozyme (90, 224–227), ovalbumin (61, 206, 279, 297), thymidine kinase (216), β-tubulin (190), vitellogenin (50, 51).
Dictyostelium: developmental gene[b] (22), cysteine proteinase (64), ribosomal RNA (62).
Drosophila: chorion loci (304), glue proteins (242–247), heat-shock loci (14, 48, 76, 79, 149, 209–213, 300), histone loci (109, 148), ribosomal protein (215), 5S RNA (169), tRNA gene cluster (298).

Table 2 (*Continued*)

Goat: β-globin-cluster (317, 318).
Hamster: dihydrofolate reductase (15, 299).
Human: α-globin-cluster (237), β-globin-cluster (16, 233–236, 238), c-*myc* (218), β-interferon (178, 301), dihydrofolate reductase (201, 319), HPRT (320), HLA-DRα (302), histone H4 [165, c-Ki-*ras* (63)].
Maize: *Adh1* (11, 166).
Mouse: acetylcholine receptor (242), complement C4 (229), α-globin (303), β-globin (17, 75, 250, 251), collagen (53), G6PD (321), HPRT (321), Igκ (285), metallothionine (214).
Neurospora: qa cluster (23, 23a).
Pisum: ribosomal RNA[b] (13).
Rat: cytochrome P-450c (382, 383), α-fetoprotein (249), elastase I (252), insulinlike growth factor II (314), preproinsulin (18), prolactin (305), serum albumin (248, 249), tyrosine aminotransferase (222), tryptophan oxygenase (222).
Saccharomyces cerevisiae: ADC1 (26), *ADR2* (26), *GAL1–GAL10* (38, 39, 42, 69, 69a, 72, 289), *HIS3* (43), *HSP82* (45, 46), *MAT* loci (25), *PHO5* (40, 41, 47, 232), *TRP1* (37, 44, 292–294), ribosomal RNA (322), *URA3* (44).
Sea urchin: histone loci (19, 306).
Tetrahymena: histone H4 (307), 5S RNA (308), ribosomal RNA (20, 192).
Xenopus: β-globin (54), histone H4 (309), 5S RNA (310), ribosomal RNA (21).

E. Transcription terminators[b]
Bombyx: fibroin H-chain (381).
Chicken: α-globin-cluster (8, 89), β-globin (8, 277), lysozyme (90, 224–227), ovalbumin (207), vitellogenin (50).
Drosophila: chorion loci (304), heat-shock loci (14), Sgs-4 (242).
Human: α-globin-cluster (237), β-globin-cluster (233).
Saccharomyces cerevisiae: GAL1–GAL10 (42), *HSP82* (45, 46), *MAT* loci (25), *PHO3/PHO5* (47), *TRP1* (44), *URA3* (44).
Sea urchin: histone cluster (19, 306).
Xenopus: histone H4 (309).

II. Replication origins

A. Viral

Bovine Papilloma Virus (28).
Polyoma (33, 275, 276).
SV40 (9, 10, 34, 54, 80, 119, 186–189, 204, 257–274).

B. Episomal

Saccharomyces cerevisiae: two-micron circle (35, 311), *TRP1ARS1* (44, 292–294).
Tetrahymena: ribosomal RNA (20, 192).

III. Topoisomerase cleavage sites

A. Topoisomerase I

Dictyostelium: ribosomal RNA (312).
Tetrahymena: ribosomal RNA (65, 127, 129).

B. Topoisomerase II

Drosophila: heat-shock loci (134, 137), histone gene cluster (134).
SV40: (133).

IV.[c] Recombinational elements

Mouse: IgH switch (313).
Saccharomyces cerevisiae: two-micron circle *FLP* sequence (35), *MAT* loci Y-Z junctions (25).

V. Centromeres

Saccharomyces cerevisiae: CEN3 (24, 315), *CEN11* (24, 36, 316).

VI. Telomeres

Tetrahymena: ribosomal RNA (463).

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[a] Listing compiled in August 1987.
[b] presumptive functional sequence assignment

MspI fragment that migrated on nucleoprotein gels like protein-free DNA. In the case of SV40 minichromosomes, the hypersensitive site looks like extended B-form DNA under the electron microscope (186–189, 260, 269), but DNA-bound globular particles along with T-antigen can be detected within this region in a subfraction of these molecules (189), as well as barriers to Bal-31 (80), footprints to DNase I (180), and resistance to psoralen crosslinking (173). Thus, it seems likely that this region is histone-free but associated with a number of transcription factors (see below). Furthermore, Solomon & Varshavsky (204) have demonstrated that formaldehyde, which preferentially crosslinks histones to DNA, fails to crosslink any proteins to the SV40 nuclease hypersensitive region in living cells. In addition, no histone crosslinking could be detected to DNA within the 5'-hypersensitive sites of *Drosophila* hsp70 genes (203). Thus, all available evidence to date suggests that histones are not present within hypersensitive sites.

Topoisomerases I and II

Topoisomerase I appears to be present in a specific subset of hypersensitive sites. Employing sodium dodecyl sulfate lysis of nuclei to cause in situ DNA cleavage at the positions of topoisomerase I interaction, the enzyme has been localized in the replication origins and promoters of the extrachromosomal ribosomal RNA genes of *Tetrahymena* (127, 129, 324) (see also Ref. 312). Interestingly, cleavage occurs specifically at the same 16-bp conserved consensus sequence in chromatin as in naked DNA, indicating that the sequence itself has a high affinity for topoisomerase I (65, 127, 129, 132). However, in the class II chromosomal genes that have been studied, which include *Drosophila* heat shock and copia, and rat tyrosine aminotransferase (128, 131, 323), topoisomerase I is preferentially localized within the active transcription units and is absent from the upstream hypersensitive sites. Although the drug camptothecin was used in two of these studies (128, 131) and has the potential to lead to artifacts (see below and Ref. 65), photo-crosslinking of living cells yields conceptually similar results (323). Thus, the location of topoisomerase I within hypersensitive sites may be a peculiarity of class I genes or small linear extrachromosomal templates (or both).

Prominent endogenous topoisomerase II cleavage sites have been detected near the nuclease hypersensitive site in SV40 minichromosomes (133), and within the 5'-hypersensitive sites of the *Drosophila* hsp70 genes (134, 137). Since drugs were used in these studies to induce protein-linked DNA cleavage, the question is raised: Are the endogenous enzymes normally present within these hypersensitive sites or are the drugs simply trapping occasional molecules that are transiently inspecting accessible regions in chromatin? Another potential complication is that the position of topoisomerase II cleavage can be modulated depending on what drug is used (66). Therefore, more

definitive approaches, perhaps involving photo-crosslinking procedures (202, 323), are necessary to demonstrate the in vivo association of topoisomerase II with nuclease hypersensitive sites. It is significant that in somatic vertebrate cells this enzyme is a marker of proliferation and essentially disappears when cell division ceases, even in transcriptionally active tissues like liver (325). Thus, topoisomerase II may play roles in chromatin assembly, DNA replication, and decatenation of chromosomal loops (325, 325a). Hypersensitive sites with which this enzyme may be associated are likely to be functionally linked with these processes.

RNA Polymerase II

An intriguing observation made possible by the UV-crosslinking experiments of Gilmour & Lis (202) is that RNA polymerase II is localized in the 5'-nuclease hypersensitive site of the uninduced *Drosophila* hsp70 gene. Such resting polymerases may reflect mechanisms of poising chromatin for future transcription or indicate a previously active state. Using run-on transcription, RNA polymerase II has also been detected clustered at the 5'-end of the nontranscribed β-globin gene in chicken erythrocyte nuclei (326). Taken together, these findings suggest that the promoter regions of many other genes may also have resting RNA polymerase molecules.

Transcription Factors and Other Proteins

Most hypersensitive sites possess internal "cold" spots and/or exonuclease barriers that indicate the presence of bound proteins (39, 46, 69–74, 76, 80, 98, 102, 165, 166, 178, 183, 201, 289, 295, 318), yet identification of the specific proteins that are responsible for such protection has largely been by implication. Much progress has been made on the in vitro characterization of transcription factors and the DNA sequences to which they specifically bind (for reviews, see Refs. 327–331). Often the regions in naked DNA that are footprinted by such factors correspond to protected regions within nuclease hypersensitive sites in chromatin. This correlation holds for the TATA-binding protein(s) and heat-shock transcription factor (HSTF) in *Drosophila* heat-shock loci (76, 98–101, 332, 333), for Sp1 in SV40 (180, 328), for the glucocorticoid receptor in the rat tyrosine aminotransferase gene (73), for nuclear factor-1 (NF-1) or the CCAAT-binding transcription factor (CTF) in the long terminal repeat of the mouse mammary tumor virus (102, 334, 335), and for the AAGATAAGG-binding factor of the chicken β-globin gene promoter (295) (see also Refs. 95, 183, 336, 337). The most definitive studies have employed either immunological or genetic approaches. Immunoelectron microscopy localizes T-antigen near the border of the nucleosome-free region in SV40 minichromosomes (189), while gene disruption experiments in yeast strongly implicate GAL4 as the factor that footprints regions within the

hypersensitive site of the upstream activating sequence, UAS_G (39, 69, 69a, 72, 289). However, in many other cases the proteins responsible for mediating protection in hypersensitive sites have yet to be characterized (24, 70, 71, 74, 165, 166, 178, 201). Promoters and enhancers often possess multiple, and sometimes overlapping, sequence motifs that each can interact with distinct *trans*-acting factors (327–331, 372–376). The question of which combination of factors is present within hypersensitive sites in vivo will therefore remain quite challenging. The UV-crosslinking procedure of Gilmour & Lis (202, 323) is one appropriate way to address this issue.

MECHANISMS OF ESTABLISHMENT OF HYPERSENSITIVE SITES

cis-*Acting DNA Sequence Determinants*

The hypersensitivity of a site is often determined by its underlying or flanking DNA sequence. Appropriate tissue-specific hypersensitive sites are generated, for example, following integration of β-globin (250, 251) or elastase I (252) *trans*-genes into the mouse germ line. In the case of elastase I, the 134-bp tissue-specific enhancer generates a local DNase I–hypersensitive site and activates transcription when moved either upstream or downstream of the promoter (252). In *Drosophila*, P-element–mediated germ line transformation reveals that introduced hsp28 (300) or glue protein genes (245) generate hypersensitive sites in the appropriate 5'-promoter regions. This general observation is also true for a variety of other genes stably integrated into cultured mammalian cells (271, 291, 301, 319), for plasmids introduced into yeast (24, 40, 210, 213), and for sequences microinjected into *Xenopus* oocytes (54, 309). Furthermore, mutational analysis of hypersensitive regions directly demonstrates that sequences corresponding to binding sites for *trans*-acting factors are necessary for generating hypersensitivity. This is true in the case of the SV40 72-bp and 21-bp repeat elements and origin of replication (188, 259, 268, 269, 274), which respectively bind enhancer factors, Sp1, and T-antigen (327, 328, 372–375). This also may be true for several *cis*-acting elements in polyoma virus (275, 276, 375, 376).

Sequences that immediately flank *cis*-acting elements also can contribute to or exhibit hypersensitivity. In SV40, sequences next to the enhancer and 21-bp repeats have been shown to affect the generation of the nucleosome-free region, suggesting that multiple sequence motifs are required to generate a stable site (188, 268, 274). Introduction of *Drosophila* hsp70 gene constructs into yeast has revealed that the HSTF-binding site is important for generating hypersensitivity on immediately adjacent 5'-sequences, even when these flanking sequences are of foreign origin (213). In *Drosophila*, this constitutive hypersensitive site becomes associated with HSTF only after

induction (98–101), but in yeast HSTF may be bound even before heat-shock (45, 46, 371, 371a). Similarly, 5' and 3' hypersensitive sites flanking yeast *CEN3* are maintained even on substituted foreign sequences (36). In summary, it appears that several simple mechanisms may operate to generate hypersensitive sites that depend, in part, on the single or multiple binding of *trans*-acting factors.

Sequences that generate hypersensitive sites can sometimes induce or suppress the formation of other hypersensitive sites even over long distances. This phenomenon has been observed for the SV40 enhancer (54), retroviral long terminal repeats (31, 53, 217, 338), silencers in the yeast mating type loci (25), and various upstream sequences in the *Drosophila* Sgs-4 gene (242, 243). Cooperative communication between distally located sequences has also been noted for rat glucocorticoid response elements (GREs) (223, 351). Functional interaction between hypersensitive sites might come about by looping, torsional stress, cooperative spreading of specific chromatin structures along a fiber, or modulation of DNA methylation patterns (see below).

trans-*Acting Protein Factors*

INDUCIBLE SITES The role of *trans*-acting factors in generating localized nuclease hypersensitivity is perhaps best understood for hormone-inducible sites that otherwise exist as canonical nucleosomal structures. The promoter region within the long terminal repeat (LTR) of the mouse mammary tumor virus (MMTV) contains a glucocorticoid-responsive element (GRE) that triggers transcriptional initiation within a minute after exposure of cells to hormone (352–354). Prior to induction, sequence-positioned nucleosomes are organized along the LTR (191); in response to hormone, a nuclease-hypersensitive site is generated (31, 102, 142, 191) that corresponds to the displacement (or alteration) of a single nucleosome from the GRE region (-60 to -250) (191). Induction of the site is blocked by an antagonist that covalently modifies the receptor (355), consistent with in vivo footprinting experiments that suggest that receptors bind to GREs only after hormone treatment (73). [This is in contrast to in vitro binding results (370) and may be due to the regulated in vivo association of receptor with another protein (462)]. Interestingly, induction of the site is accompanied by the binding of NF-1 and another factor(s) to the -82 to +12 region (102). The entire process is reversible as the hypersensitive site disappears rapidly upon removal of hormone (31). Thus, induction occurs by the successive binding of hormone to receptor, hormone-receptor complex to the GRE, loading of NF-1 and another factor(s), and an accompanying loss of nucleosomal structure. These observations imply that even though the GRE is localized in a nucleosome prior to induction, the sequence must be highly accessible for interaction with the hormone-receptor complex and hence possesses a nucleosome phasing

frame poised for transcription. An interesting question is whether mutations in the NF-1 binding site, which are known to strongly impair transcriptional induction (334), will also affect the inducibility of the nuclease-hypersensitive site. Clearly, one would predict that mutations in the GRE should prevent the site from forming in response to hormone.

TISSUE-SPECIFIC SITES The chicken adult β-globin gene possesses a 200-bp hypersensitive site in the 5' promoter region in red blood cells, but a nucleosomal organization in other tissues (239). The hypersensitive site can be reconstituted in vitro onto cloned DNA templates provided that nuclear extracts containing nonhistone proteins from red blood cells are present prior to or during nucleosome assembly, but not after (52). More recently, a series of factors that footprint specific sequences in this 5'-region (95, 295, 336, 337) has been identified. Presumably the binding of some combination of these factors is responsible for generating this tissue-specific hypersensitive site by preventing nucleosome formation.

Similarly, transcriptionally active templates can be generated by in vitro reconstitution experiments employing *Xenopus* 5S cloned genes if transcription factor TFIIIA is present prior to or during nucleosome formation, but not after (356–360). Other studies as well indicate that nucleosome formation preferentially inhibits transcriptional initiation (361–363). Replication may thus be necessary in these cases to disrupt histone-DNA interactions to allow transcription factor penetration. The TFIIIA binding site in the *Xenopus laevis* somatic 5S RNA gene is on the same helical face that interacts with the histone octamer (360), and thus can only bind to either histones or TFIIIA but not to both. However, a different phasing frame exists for the *Xenopus borealis* 5S gene, which can partially bind TFIIIA after nucleosome assembly (364). In addition, heterokaryon experiments reveal that many silent genes can be activated in the absence of DNA synthesis (365–367).

DEVELOPMENTAL SITES SV40 possesses a developmentally regulated hypersensitive site. Encapsidated genomes lack this site (119, 377–380), whereas >95% of the chromosomes that accumulate in mutants blocked in virion assembly possess the site (119). What causes the hypersensitive site to disappear during encapsidation? Does an additional nucleosome bind to cover the hypersensitive region or do the preexisting nucleosomes simply slide to new positions? When only a minor fraction of SV40 minichromosomes possess the hypersensitive site, the average nucleosome repeat length of the population is 198 bp; on the other hand, when nearly every molecule in the SV40 minichromosome population possesses the site, the repeat length is 177 bp (368). Since the number of supercoils and hence the presumed number of nucleosomes appears to be similar between these two populations of mole-

cules (130, 368), the sliding model seems most likely (369). Sp1, T-antigen, and enhancer-binding proteins (327, 328, 372–375) are probably important in preventing nucleosome coverage of this region, as demonstrated by mutational (188, 259, 268, 269, 274), immunological (189), and genomic footprinting experiments (180), whereas the major capsid protein, VP1, has been implicated in triggering nucleosome sliding over this site during encapsidation (368, 369). An interesting question, not yet addressed, concerns the temporal appearance of the site after viral penetration into cells. Thus far only replicating and postreplicated SV40 minichromosomes have been studied (119, 263, 368). Presumably the site is generated prior to replication by a reversal of the nucleosome sliding process that closed the site. Clearly, it should be possible to test these ideas with purified proteins and minichromosomes in vitro.

CONSTITUTIVE SITES While inducible, tissue-specific, and developmental hypersensitive sites are organized into nucleosomes in some circumstances, this may not be the case for constitutive sites. As previously discussed, before transcriptional activation the promoter region of the *Drosophila* hsp70 gene is nuclease hypersensitive and is believed to be associated with TATA-binding protein(s) (98–101) and RNA polymerase (202); HSTF binds only after heat-shock induction (98–101). It has been proposed that the TATA-binding protein(s) create this hypersensitive site (98). In yeast, however, HSTF appears to be bound prior to induction (45, 46, 371, 371a), and therefore could play a role in hypersensitive site formation. Obviously, these possibilities are directly testable, for example, by mutagenesis of the appropriate sequences and site-directed integration. Other constitutive sites may not require the preexisting association of DNA-binding proteins to be hypersensitive; in order to fulfill their functions they may need to always be nucleosome-free to interact with *trans*-acting factors. A case for this point comes from elegant experiments in *Saccharomyces cerevisiae* that demonstrate that the hypersensitive site in the upstream activating sequence (UAS_G) in the *GAL1-GAL10* locus persists in the combined absence of galactose and the *trans*-acting factors encoded by *GAL4* and *GAL80* (39, 289; D. Lohr, J. E. Hopper, personal communication). In fact, footprinted subdomains within UAS_G appear only upon binding of the *GAL4* protein (39, 69, 69a, 72). Whether a special class of *trans*-acting factors is responsible for generating and maintaining this and other constitutive sites remains to be determined, as well as the interesting possibility that *GAL4* is necessary only for the generation, but not for the maintenance or propagation, of this site. Alternatively, these sites may possess DNA sequences that inherently tend to exclude nucleosomes in the absence of *trans*-acting factors, or may be flanked by strong nucleosome phasing sequences, leaving a space too small within the site itself (<146 bp) to accommodate a nucleosome (see below).

DNA Conformation

Persuasive evidence exists indicating that the conformation of DNA within hypersensitive sites is atypical. It is particularly striking that in many cases the same sequences in nuclei and in naked supercoiled DNA are hypersensitive to agents that recognize non–B-form DNA structures and unpaired bases (115, 120, 143, 212), including Bal-31, S1, mung bean, and *Neurospora crassa* nucleases and bromoacetaldehyde (see Table 1). These observations raise a number of interesting questions: Is torsional stress responsible for the assembly of hypersensitive sites? What is the helical conformation of DNA within such regions? How might the binding of nonhistone proteins either cause, or be affected by, such altered DNA conformations?

A role for torsional stress in transcriptional activation and hypersensitive site formation is suggested by the following observations. In avian erythroid cells, the general increase in DNase I sensitivity seen throughout the transcriptionally active β-globin gene chromatin domain is reversed by agents that relax DNA in vivo (55, 56). Similarly, transcriptionally active 5S RNA gene-containing plasmids are assembled into torsionally stressed, "dynamic" minichromosomes in *Xenopus* oocytes (310, 359, 385). Furthermore, the preferential nuclease sensitivity and preferred cutting at the nucleosome-free region in SV40 requires torsional stress under some experimental conditions (261, 264–266). Whether this stress is the cause or effect of hypersensitive site formation remains to be determined. In many cases, it is possible that torsional stress is the consequence of hypersensitive site formation, since the generation of a hypersensitive site through histone octamer displacement would liberate approximately two unconstrained superhelical turns of DNA. The resulting elastic strain would simply represent the "memory" of the displaced histones. However, unrestrained DNA supercoils can even be generated in the absence of octamer displacement, since when nucleosomal DNA is subjected to extreme torsional stress with DNA gyrase, the histones can remain bound but are unable to constrain any DNA supercoils (386). Stress can facilitate mini-loop formation and generate stable hypersensitive sites in model prokaryotic systems (348, 349). On the other hand, protein binding can induce torsional strain by causing DNA to bend (347). Stress may be localized within hypersensitive regions or be intermingled with torsionally constrained (i.e. nucleosome-associated) supercoils (264, 388), as opposed to being spread throughout an entire minichromosome (350).

DNA sequences within some hypersensitive sites have been shown to disfavor nucleosome assembly and to dissociate histone octamers if mildly perturbed (52, 115, 387). Interestingly, polypurine-polypyrimidine tracts are often found in promoter regions (199, 344–346, 384) and are known to "repel" histones in in vitro reconstitution experiments (339, 340); tracts longer than 80 bp do not assemble nucleosomes (341). Right-handed A-form

helixes, such as double-stranded RNA (341), RNA/DNA-hybrids (342), and ribosubstituted DNA (343), and left-handed Z-form DNA (391) similarly are unable to participate in nucleosome formation. Thus, it appears that local perturbations in DNA helical pitch, flexibility, handedness, and bending play crucial roles in nucleosome assembly. In supercoiled plasmids, polypurine-polypyrimidine tracts are hyperreactive to probes that specifically detect non–B-form DNA (114, 121, 123, 144, 199, 346, 384, 389, 390). A variety of models have been proposed for the helical conformation of these tracts, including a change in handedness (389), DNA slippage (199), radial asymmetry (144), and Hoogsteen or reverse Hoogsteen base pairing (123).

Even though sequences have the potential to assume conformations unfavorable for nucleosome assembly in vitro, they do not necessarily do so in vivo. For example, the ubiquitous, potential Z-forming eukaryotic-specific sequence $d(CA)_n \cdot d(GT)_n$ does not flank S1 nuclease hypersensitive sites (81, 392) and is packaged into canonical nucleosomes in a B-form–like structure (81, 393; see Ref. 403 for review).

In summary, DNA conformation dictated by the primary sequence per se, or induced by torsional stress or protein binding, must be fundamentally important in hypersensitive site formation. A testable hypothesis is that nucleosome formation is prevented by the combination of short stretches of conformationally altered DNA working synergistically with the binding of nonhistone proteins to adjacent typical B-form sequences. Alternatively, the binding of specific *trans*-acting factors to unusual helical structures—such as polypurine-polypyrimidine tracts or "bent" DNA (464, 465)—might induce the spreading of a non–B-form structure to flanking sequences and thereby exclude nucleosomes.

MAINTENANCE AND PROPAGATION OF HYPERSENSITIVE SITES

Little is known about the mechanisms that maintain and propagate hypersensitive sites. The now classic experiments of Groudine & Weintraub reveal that induction of a hypersensitive site does not necessarily require protein synthesis (220). Once formed, sites can be propagated for at least 20 generations, even in the absence of an associated function (220). They proposed that hypersensitive sites can template their structure to daughter cells through a DNA replication process independent of the initial inducing stimuli but related to their single-stranded character (220). Even if sites prove not to have bona fide single-stranded DNA but instead alternative non–B-duplex structures (see above), the conceptual framework of this model may still be essentially correct. Perhaps maintenance proteins exist to aid in the templating of sites through DNA replication to daughter cells. It should be noted,

however, that in vitro experiments have failed to provide evidence for such a templating process (441).

What other mechanisms might operate to propagate hypersensitive sites? Active genes are located near the nuclear periphery and thus may be advantageously situated to optimally interact with regulatory proteins upon nuclear entry from their cytoplasmic site of synthesis (443). In addition, active genes replicate in early S phase, in contrast to inactive sequences (394–396). It has been proposed that crucial transcription factors, which are believed to exist in limiting amounts, would be titrated by the early replicating sequences and thereby provide a mechanism for the inheritance of specific chromatin structures, such as hypersensitive sites (356, 357, 397). However, in the case of SV40, where the hypersensitive site can be followed during the DNA replication process, there is a lag in the appearance of the site in the replicated molecules (263). Since the nucleosome-free region in SV40 also corresponds to the origin of replication, presumably a multiprotein complex is transiently associated with this site during DNA synthesis and accounts for this observation.

ROLE OF DNA METHYLATION

The modified base, 5-methylcytosine, found in vertebrate DNA nearly exclusively in the dinucleotide sequence CpG and constituting 2–7% of all cytosine residues, has been implicated as an epigenetic element in the repression of gene activity (reviewed in Refs. 398–403). Eukaryotes with small genome sizes, however, such as yeast (439) and *Drosophila* (440), lack this modification. Nevertheless, in more complex organisms, a positive correlation exists between hypomethylation of promoter-proximal CpG sequences, associated nuclease hypersensitive sites, and gene transcription (29, 50, 89, 291, 319, 404, 405, 407, 411, 422). Does DNA methylation, therefore, play a direct role in regulating the formation of hypersensitive sites?

Several lines of evidence suggest that DNA methylation suppresses hypersensitive site formation. Proviral induced de novo methylation of the murine α-1 (I) collagen gene is correlated with transcriptional inactivation and elimination of a transcription-associated hypersensitive site, while strikingly, two other, nontranscriptionally related sites, are spared (53, 409, 410). Transfection experiments reveal that in vitro methylation of CpG sequences in either promoter or coding regions is sufficient to repress expression of most transgenes (414–417, 421). Cedar and coworkers (413) further showed that this transcriptional repression is accompanied by assembly of the transfected DNA into nuclease-resistant chromatin structures. Interestingly, when the "housekeeping" gene, dihydrofolate reductase, is similarly methylated in vitro and transfected into CHO cells, site-specific demethylation in the vicin-

ity of the transcription start site, hypersensitive site formation, and transcriptional activation occur (319). Likewise, when a fully methylated skeletal muscle α-actin gene was transfected into myogenic cells (as opposed to fibroblasts) it was demethylated and expressed (418). Similar site-specific demethylation of a murine proviral enhancer element has also been seen (408). These observations suggest that tissue-specific *trans*-acting protein factors can trigger demethylation. However, whether demethylation occurs before or after hypersensitive site formation is not known.

On the other hand, hypomethylation appears to create a permissive environment for establishing hypersensitive sites (29, 291, 407, 411, 422). During late spermatogenesis in birds and mammals, a subset of CpG residues associated with many constitutively expressed housekeeping genes is selectively undermethylated and apparently excluded from the wave of de novo methylation that occurs during gamete formation (402, 407). Groudine & Conklin (407) have established a link between the location of undermethylated CpG sequences within constitutively expressed genes in chicken sperm DNA and the subsequent appearance of nuclease hypersensitive sites in somatic cells of the next generation, suggesting that constitutive paternal genes are programmed for expression in the early avian embryo by undermethylation.

An experimental limitation in most studies on DNA methylation has been that methylation-sensitive restriction endonucleases, such as Hpa II or Hha I, only detect one-sixteenth of all possible methylated CpG sites. Furthermore, many such enzymes are incapable of distinguishing between hemimethylated and fully methylated sites. Jost and coworkers (411) circumvented these problems by using genomic sequencing, and made the striking observation that demethylation paralleling transcriptional activation occurs on only one strand of the double helix in the hormone-inducible hypersensitive site of the avian vitellogenin gene. Strand-specific demethylation probably occurs by a novel excision-repair mechanism, whereby a 5-methylcytosine residue is enzymatically removed and replaced with cytosine (412). These observations underline the potential serious weaknesses in previous studies that have failed to observe a correlation between demethylation and the establishment of hypersensitive sites (e.g. Refs. 17, 420). Nonetheless, it is clear from the experiment of Macleod and Bird (423) that undermethylation by itself is insufficient to trigger the formation of hypersensitive sites and to activate transcription; perhaps specific *trans*-acting factors and/or flanking chromatin structures also play significant roles. In addition, de novo methylation seems to be the consequence, and not the cause, of X-chromosome inactivation (424), possibly reflecting its importance primarily as a maintenance mechanism.

In summary, demethylation of CpG residues in promoter-proximal regions seems to be necessary but not sufficient for the establishment of nuclease

hypersensitive sites. Likewise, methylation of these sequences often appears to suppress the formation of hypersensitive sites. There are several experimentally testable mechanisms by which DNA methylation could mediate these effects: (*a*) Alteration of the structure of B-form DNA. In the most extreme case, methylation can promote a *B* to *Z* transition (442) (see Ref. 403 for review). However, methylation of CpG sites has no discernible effect on DNA conformation in the chicken β-globin gene 5'-region over a wide range of negative superhelical densities (390). (*b*) Altering the binding affinity of *trans*-acting factors. For example, introduction of two methyl groups into the major groove of duplex DNA can significantly increase the affinity of *lac* repressor to bind to a mutant *lac* operator sequence (419), or markedly decrease the affinity of certain restriction enzymes to bind to their recognition sequences (425). It is to be appreciated that many nonhistone proteins appear to interact with the major groove in vivo (70–74, 164–166, 178). (*c*) Changing the sequence-specific positioning of nucleosomes, possibly by altering DNA bendability (see below) or by inducing histone H1 or nonhistone proteins to bind to preferred locations (466). (*d*) Increasing the binding affinity for histone H1, leading to the cooperative spreading of chromatin condensation, which is known to be mediated by this protein (467–469). Greater than 80% of methylated CpG residues are packaged in H1-containing nucleosomes (406). Formation of higher-order chromatin structures mediated by histone H1 condensation would render resident sequences inaccessible to *trans*-acting factors. (*e*) Triggering the association of methylation-specific nonhistone DNA-binding protein(s) that may serve to repress the genome (426).

NUCLEOSOME POSITIONING AND HYPERSENSITIVE SITES

Nucleosomes sometimes adopt precise, well-defined locations with respect to the underlying DNA sequence. This positioning, or "phasing," is particularly evident in regions flanking hypersensitive sites (14, 24, 25, 36, 40–42, 44, 46, 47, 75, 76, 109, 191, 192, 201, 211, 292, 316, 463) (see Figure 1D). Phasing may have important biological consequences because it can determine the availability or activity of *cis*-acting sequences in three ways: (*a*) it could bring two short sequences that are separated by the equivalent of one superhelical turn of DNA (ca. 83 bp) into close lateral proximity by assembling them into the same, rather than into neighboring nucleosomes (431, 466); (*b*) it could be responsible for defining the rotational orientation of the major and minor grooves relative to the histone octamer; and (*c*) it could place a sequence within an accessible linker region between adjacent nucleosomes.

The most common experimental approach for detection of nucleosomal

positioning employs indirect end-labeling. However, this method suffers from its inability to quantitate the fraction of phased nucleosomes along a sequence; positioned nucleosomes constituting only a minor fraction of the population can appear dominant over a larger fraction of randomly located particles. Similarly, multiple phasing frames are difficult to detect, and the sequence specificity of certain DNA cleavage reagents, like micrococcal nuclease, can be problematic (106, 107, 472). Therefore, the optimal approach has been to use a combination of nonspecific cleavage agents (e.g. DNase I, MPE) along with restriction endonucleases.

Two mechanisms have been proposed to account for nucleosome positioning, termed here the "passive" and the "active" models. The passive model views the location of nucleosomes as purely statistical, or random, with short-range order conferred by the presence of protein-DNA complexes that exclude nucleosomes, creating "boundary" conditions (427). The active model proposes that nucleosome cores occupy distinct positions along DNA because of the underlying sequence (428). It is now clear that both of these mechanisms operate to phase nucleosomes, but to different degrees depending on the system.

Indirect support for the passive or boundary exclusion model comes from the observation that extended arrays of phased nucleosomes often exist between two nuclease hypersensitive sites, in yeast (25, 41, 46, 47), *Drosophila* (76, 109, 211), and *Tetrahymena* (192, 463). In addition, phased arrays of nucleosomes have been observed to spread inward from the telomeres of *Oxytrichia* (193) and *Tetrahymena* (463) chromosomes, exhibiting the sharpest phasing closest to the chromosome ends (193) in agreement with the prediction of the statistical model (427). Direct support for the passive model comes from experiments that mutationally delete hypersensitive sites and demonstrate the concomitant loss of nucleosome phasing on flanking sequences, as seen for yeast *PHO5* (40) and *TRP1* (44) genes. However, these results could be taken to formally support yet another model, in which the hypersensitive site initiates a cooperative spreading event that is unidirectionally transmitted along a fiber by nucleosome-nucleosome interactions (46).

In contrast to the passive model, which may apply in some circumstances, much direct evidence has been obtained for the active model of nucleosome positioning. Certain sequences precisely phase nucleosomes upon in vitro reconstitution (360, 364, 429, 430, 432–438, 461). In the case of a 5S RNA gene sequence, the same in vitro phasing frame is also established in vivo (292). In addition, deletion of the hypersensitive sites flanking the phased nucleosome arrays in yeast *CEN3* (24) or *URA3* (44) does not lead to loss of nucleosome positioning, while insertion of the *CEN3* hypersensitive site fails to confer phasing onto foreign flanking sequences (36). Therefore, underlying

DNA sequences themselves often have the capacity to phase nucleosomes.

The mechanism of active phasing is related to both the bendability of DNA molecules (428, 436, 470, 474) and to sequences located close to both sides of the central dyad axis of the nucleosome (432–434, 474). Tracts of AAA or TTT are preferentially placed where the minor groove faces inward toward the histone octamer, whereas GGG or GCC in the minor groove prefer to face outward (reviewed in Refs. 428, 436). This anisotropic flexibility seems to determine the rotational setting of DNA about the nucleosome, while the translational setting or phasing frame, i.e. the precise sequences that enter and exit the nucleosome, seems to be most sensitive to the sequences that are present near the central dyad (432–434, 474).

The active mechanism of nucleosome positioning may work in concert with—or even regulate—the process of hypersensitive site formation. For example, the *Xenopus laevis* (360) and *borealis* (364) 5S RNA genes differ in their ability to bind TFIIIA as a consequence of possessing different phasing frames. One prediction is that hypersensitive sites that are capable of being formed in the absence of DNA synthesis, through interactions of nucleosomal DNA with *trans*-acting factors, must possess phasing frames that place recognition sequences either facing outward from the histone octamer or within the linker region between two nucleosomes. As previously discussed, the hormone-inducible nuclease hypersensitive site found within the MMTV LTR presumably places the GRE in such a transcriptionally poised position (102, 191). Similarly, the mouse β-globin gene possesses phased nucleosomes from $^{-}3000$ to $+1500$ in nonerythroid cells. The identical phasing frame is retained in erythroid cells, but the nucleosomal array is interrupted by a 700-bp gap, spanning $^{-}200$ to $+500$, from which four nucleosomes have been removed (or modified) (75). Thus, for both inducible and tissue-specific hypersensitive sites, it is probable that the precise nucleosomal positioning seen both before and after transcriptional induction is mediated actively by the underlying DNA sequences. Such positioning mechanisms may have specifically evolved in eukaryotes to facilitate regulation of gene expression while minimizing a major reorganization of nucleosome arrays along a chromatin fiber, and may be related to the striking structural polymorphisms observed in intergenic DNA (107, 471–473).

CONCLUDING REMARKS AND PROSPECTS

We have seen that nuclease hypersensitive sites correspond to a highly selective fraction of the genome: crucial *cis*-acting DNA sequences that regulate chromosome structure and function. These windows presumably reflect the outcome of an evolutionary process that has the overall effect of reducing the genome size of a eukaryote to that of a prokaryote by removing

from the environment, through nucleosome assembly, competitor DNA sequences. It is of obvious interest in the future to determine how the emergence of hypersensitive sites is regulated during gamete formation, development, and differentiation, leading to unique combinations of these elements in different cell types.

Nuclease hypersensitive sites probably exert their corresponding functions by a regulatable chain of events. Generation and/or maintenance of an open region may require the association of a specific or general *trans*-acting factor(s). The nucleosome-free region now has an enhanced accessibility to bind additional, but different *trans*-acting factors. This may lead to the formation of *recognition complexes* having high affinity and specificity for the transient association of other proteins in response to functional needs (e.g. RNA polymerases). Communication between sites at far distances may occur through looping by the gathering together of these recognition complexes through protein-protein interactions (349). Related interactions may target recognition complexes to specific nuclear locations, such as the nuclear periphery. This "compartment" is enriched in early-replicating, transcriptionally active genes (443), and may be associated with structures that interconnect with nuclear pores (452) and the nuclear matrix or scaffold (453–457). Altogether this supramolecular organization may facilitate the coordinate regulation of the synthesis, processing, and transport of RNA to the cytoplasm. Indeed, apparent nuclear targeting sequences, termed matrix association regions *(MARs)* (444, 445), or scaffold attached regions *(SARs)* (446–449), are located adjacent to a subset of nuclease hypersensitive regions (444–449). Interestingly, since *MARs* appear to also exist in association with topoisomerase II (444, 448) at chromosomal loop bases in the mitotic scaffold (450, 451), adjacent nuclease hypersensitive sites must be nested together along the mitotic chromosome scaffold. This may provide a mechanism to prevent the dissassociation of *trans*-acting factors during mitosis, sequestering them for functional emergence in daughter cells following chromosome decondensation.

Currently, not all sequences that have been localized to hypersensitive sites have recognized functions. However, experience tells us that every such site should have an important function. It is likely, therefore, that we can anticipate the discovery of new classes of *cis*-acting DNA sequences, perhaps involved in the functional punctuation of chromatin domains (458), chromosome condensation and decondensation, meiotic chromosome pairing, and processes that remain to be discovered.

Organization of functionally crucial *cis*-acting sequences into open regions in chromatin is not without cost, for it results in an increased susceptibility of these sequences to mutation. These regions appear to be preferred targets for the integration of retroviruses (459) and transgenes microinjected into fertil-

ized mouse eggs (460). By definition, they are hot spots for a wide variety of DNA-damaging agents (Table 1). It is preditable that deletions and translocations should also be favored in these regions. Thus, the genetic load of an organism may be concentrated in these sites.

The precise structure of any given hypersensitive site is not yet known, even at the rudimentary level of DNA conformation and protein composition, let alone at the three-dimensional level. These questions are difficult to definitively address at present due to technical limitations. Through the combined use of genetic and molecular techniques, however, it seems probable that specific hypersensitive sites will eventually be reconstituted in vitro from homogeneous components. Wherever possible, it will be important to prove that structural information derived from analyses of such in vitro complexes is meaningful to the in vivo situation.

Knowledge of the *cis*-acting sequences and *trans*-acting factors that generate hypersensitive sites should become available in the future for a wide variety of important sequences. This information may permit the custom design of novel functional elements that override the problems of chromosomal position effects and misregulation often observed following the integration of transgenes. New approaches for gene therapy in medicine may even evolve from some of these principles.

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